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Regulation of HSL serine phosphorylation in skeletal muscle and adipose tissue

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Watt, Matthew J., Anna G. Holmes, Srijan K. Pinnamaneni, Andrew P. Garnham, Gregory R. Steinberg, Bruce E. Kemp, and Mark A. Febbraio. Regulation of HSL serine phosphorylation in skeletal muscle and adipose tissue. *Am J Physiol Endocrinol Metab* 290: E500–E508, 2006. First published September 27, 2005; doi:10.1152/ajpendo.00361.2005.—Hormone-sensitive lipase (HSL) is important for the degradation of triacylglycerol in adipose and muscle tissue, but the tissue-specific regulation of this enzyme is not fully understood. We investigated the effects of adrenergic stimulation and AMPK activation in vitro and in circumstances where AMPK activity and catecholamines are physiologically elevated in humans in vivo (during physical exercise) on HSL activity and phosphorylation at Ser^{563} and Ser^{660}, the PKA regulatory sites, and Ser^{565}, the AMPK regulatory site. In human experiments, skeletal muscle, subcutaneous adipose and venous blood samples were obtained before, at 15 and 90 min during, and 120 min after exercise. Skeletal muscle HSL activity was increased by ~80% at 15 min compared with rest and returned to resting rates at the cessation of and 120 min after exercise. Consistent with changes in plasma epinephrine, skeletal muscle HSL Ser^{563} and Ser^{660} phosphorylation were increased by 27% at 15 min ($P < 0.05$), remained elevated at 90 min, and returned to preexercise values postexercise. Skeletal muscle HSL Ser^{565} phosphorylation and AMPK signaling were increased at 90 min during, and after, exercise. Phosphorylation of adipose tissue HSL paralleled changes in skeletal muscle in vivo, except HSL Ser^{660} was elevated 80% in adipose compared with 35% in skeletal muscle during exercise. Studies in L6 myotubes and 3T3-L1 adipocytes revealed important tissue differences in the regulation of HSL. AMPK inhibited epinephrine-induced HSL activity in L6 myotubes and was associated with reduced HSL Ser^{660} but not Ser^{563} phosphorylation. HSL activity was reduced in L6 myotubes expressing constitutively active AMPK, confirming the inhibitory effects of AMPK on HSL activity. Conversely, in 3T3-L1 adipocytes, AMPK activation after epinephrine stimulation did not prevent HSL activity or glycerol release, which coincided with maintenance of HSL Ser^{660} phosphorylation. Taken together, these data indicate that HSL activity is maintained in the face of AMPK activation as a result of elevated HSL Ser^{660} phosphorylation in adipose tissue but not skeletal muscle.

hormone-sensitive lipase; exercise; fat metabolism; AMP-activated protein kinase

ENDOGENOUS TRIACYLGLYCEROLS represent the largest fuel reserve in the body. Fatty acids derived from adipose tissue triacylglycerol and intramyocellular triacylglycerol (IMTG) lipolysis are an important energy source, but excessive accumulation of triacylglycerol is linked to insulin resistance and type 2 diabetes (2, 18). Hormone-sensitive lipase (HSL) is a major enzyme involved in triacylglycerol lipolysis, and its activity is controlled by phosphorylation in response to adrenergic and intracellular effectors. Epinephrine increases HSL activity in resting and contracting muscle (14, 16, 30) via β-adrenergic receptor stimulation and protein kinase A (PKA) activation, as it does in adipose tissue. However, unlike adipose tissue, skeletal muscle is subjected to large increases in energy demand during contractions. Skeletal muscle HSL activity is increased by 50–100% at the onset of contractions/exercise in an intensity-dependent relationship (15, 27), and activity declines toward basal rates late in prolonged moderate-intensity exercise (20, 26). In contrast, adipose tissue HSL activity (28) and lipolysis (31) remain elevated throughout exercise, suggesting tissue-specific regulation of HSL. The signaling pathways mediating the contraction and adrenergic effects on HSL are incompletely understood.

Reversible phosphorylation at several serine sites is a hallmark of HSL regulation. Studies using isolated HSL protein have demonstrated that HSL is phosphorylated on five serine residues (563, 565, 600, 659, 660; rat sequence, Fig. 1*A*) in vitro. Mutagenesis experiments demonstrate that Ser^{563}, Ser^{659}, and Ser^{660} are the major PKA phosphorylation sites responsible for stimulating HSL (1, 22), although Ser^{563} may not affect catalytic activity directly (1). Extracellular signal-regulated kinase (ERK) phosphorylates Ser^{600} and increases HSL activity in 3T3-L1 adipocytes (12), whereas phosphorylation at Ser^{565} by AMP-activated protein kinase (AMPK) and calcium/calmodulin-dependent kinase II in isolated bovine HSL prevents activation by PKA (11). The role of AMPK in controlling HSL activity and triacylglycerol metabolism has generated considerable interest because AMPK increases with exercise and phosphorylates other key enzymes in lipid metabolism. Recently, we showed that AMPK activation by 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR) administration in L6 myotubes or prior glycogen depletion in human skeletal muscle prevents the adrenergic and exercise-induced increase in HSL activity, respectively (29), whereas others found that AICAR administration blunts endogenous triacylglycerol hydrolysis in isolated contracting rat soleus muscle (23). Conversely, no changes in HSL activity were observed

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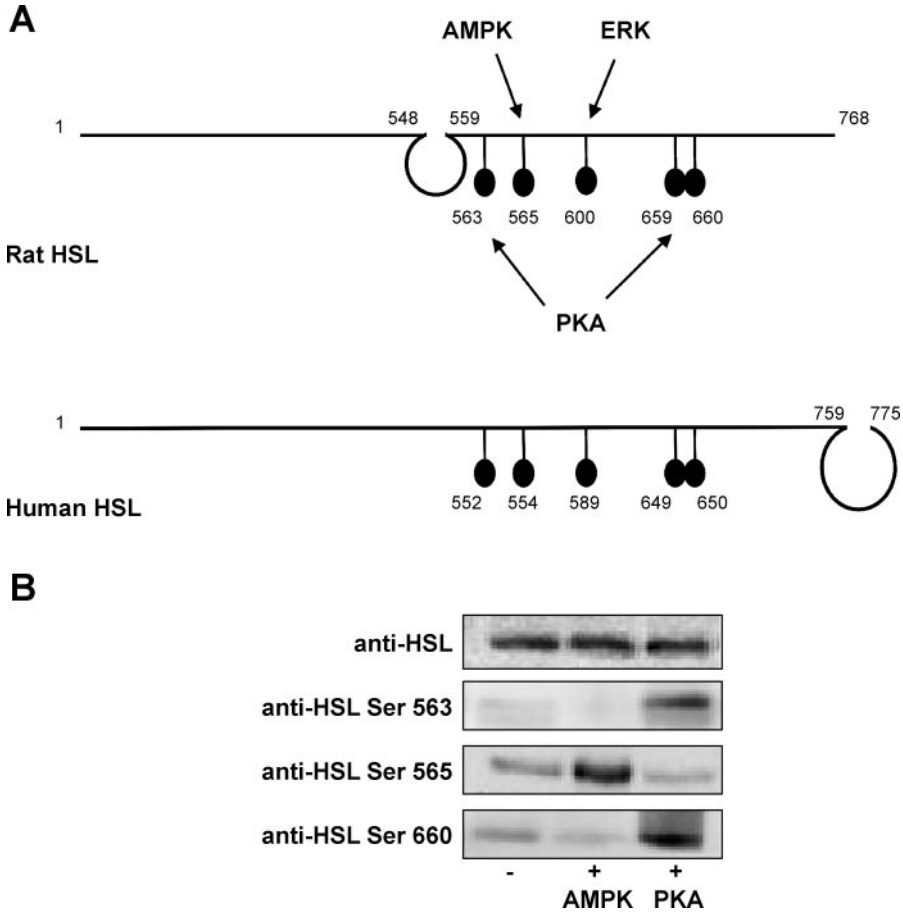


Fig. 1. *A*: comparison of rat and human hormone-sensitive lipase (HSL) sequences, shown aligned as horizontal lines, and the corresponding insert/extension sequences, represented as loops. Phosphorylation sites are shown along with the corresponding kinases using rat (P25304) and human (Q05469) sequences. ERK, extracellular signal-regulated kinase. *B*: in vitro validation of phosphospecific HSL antibodies. Purified recombinant HSL protein was incubated alone or in the presence of PKA or AMPK. Proteins were solubilized and probed with antibodies raised against anti-HSL Ser^{563}, HSL Ser^{565}, and HSL Ser^{660}. Membranes were stripped, and equal loading of total HSL protein was confirmed using an anti-HSL antibody.

when AMPK was activated and HSL Ser^{565} was phosphorylated during contractions in isolated muscle (8) and exercise in humans (20). Similarly, some studies have demonstrated decreased lipolysis in isolated rat adipocytes following AMPK activation (6, 24), whereas others have reported that PKA stimulates AMPK phosphorylation and activity (17) and that AMPK is required for maximal activation of lipolysis (34).

To investigate the tissue-specific role of HSL phosphorylation on enzyme activity, we conducted studies in humans in vivo and measured HSL activity and the phosphorylation of skeletal muscle and adipose tissue HSL Ser^{563}, Ser^{565}, and Ser^{660} at multiple time points during and after prolonged moderate intensity exercise. We then compared the effects of β-adrenergic stimulation and AMPK activation in L6 skeletal muscle myotubes and 3T3-L1 adipocytes. Our results indicated that reduced HSL activity in skeletal muscle parallels AMPK activation and HSL Ser^{565} phosphorylation, whereas adipose tissue HSL activity remained elevated, possibly resulting from increased HSL Ser^{660} phosphorylation. These findings provide an explanation for the blunting of skeletal muscle, but not adipose tissue, lipolysis during prolonged exercise.

METHODS

Human Studies

Six males and two females (23 ± 1 yr, 73 ± 7 kg, body mass index 23.1 ± 1.9 kg/m^2) participated in the study after being informed of the procedures and associated risks, which were approved by the RMIT Human Ethics Committee and were in accordance with the Declaration of Helsinki. Subjects were recreationally active and performed 2–5 exercise sessions/wk for ~60 min/session. Although sex differences in fuel metabolism exist in some instances, the measured hormonal and enzymatic responses in the present study were not different between the sexes. Thus all human data represent both male and female responses. Subjects visited the laboratory on two occasions. On the first visit, subjects completed an incremental cycling test (Lode, Groningen, The Netherlands) to volitional exhaustion for determination of their maximal pulmonary oxygen uptake ($\dot{V}O_{2\,max}$), which averaged 51.3 ± 2.4 $ml \cdot kg^{-1} \cdot min^{-1}$. Expired contents of oxygen and carbon dioxide, and ventilation were collected and analyzed on-line (Quark b2; COSMED, Rome, Italy).

At least 1 wk later, subjects returned to the laboratory at 0800 after an overnight fast, having not exercised the previous day and having consumed a 24-h standardized diet (0.2 MJ/kg body mass; 80% carbohydrate). Subjects were asked to lie supine on a bed, and a teflon catheter was inserted into an antecubital vein. A blood sample was drawn, and the line was kept patent by intermittent injection of heparinized saline. The vastus lateralis muscle was prepared for percutaneous needle biopsies by making a small incision through the skin and deep fascia under local anesthesia (1% lidocaine, no epinephrine). An incision was made for each muscle biopsy, and incisions were separated by ≥3 cm. For abdominal subcutaneous fat sampling, one incision was made ~10 cm lateral to the umbilicus under local anesthesia, and repeated samples were taken from one site with the angle of the biopsy needle changed at each sampling time. Resting muscle and adipose samples were obtained and rapidly frozen in liquid nitrogen while the subject lay on the bed.

Subjects moved to the cycle ergometer and cycled for 90 min at ~60% $\dot{V}O_{2\,max}$. Expired respiratory gases and blood, muscle, and adipose samples were obtained at 15 and 90 min of exercise in the order listed while the subject remained on the cycle ergometer. At the conclusion of exercise, subjects rested quietly on a bed, and final muscle and blood samples were obtained 2 h after exercise. The time taken to obtain all samples was <60 s. Subjects were not permitted to eat during or after exercise but were permitted to drink water ad libitum.

Cell Culture

L6 myoblasts were maintained at 37°C (95% O_2-5% CO_2) in α-modified Eagle's medium (α-MEM) containing 10% fetal bovine serum (FBS) culture medium and 1% penicillin-streptomycin. Differentiation was induced by switching to medium containing 2% horse serum when the myoblasts were ~80% confluent. Experimental treatments were started after 4 days, by which time nearly all of the myoblasts had fused to form myotubes. Cells were then infected with a constitutively active AMPK adenovirus (100 pfu/plate) or control vector (AdGo, 100 pfu/plate) as described (32). Expression levels of both control and constitutively active (CA)-AMPK-infected cells were determined by visual examination of green fluorescent protein under ultraviolet light. Seventy-two hours after infection, ~85% of all myotubes were infected in both control and CA-AMPK-infected cells (data not shown). 3T3-L1 fibroblasts were grown to confluence in DMEM-F12 in 5% FBS. Medium was removed and differentiation medium added [DMEM-F-12 in 5% FBS containing Actrapid insulin (0.5 mU/l; Novo Nordisk), 0.1 μg/l dexamethasone, and 25 μg/l 3-isobutyl-1-methylxanthine]. Differentiating medium was removed after 3 days and replaced with DMEM-F-12 in 5% FBS with 0.5 mU/ml Actrapid insulin. The medium was then replaced every 2 days until lipid droplets were present in cells. Lipid loading typically took 48–72 h and was confirmed in preliminary experiments using Oil Red O staining (33). For both adipocytes and myotubes, cells were serum starved for 4 h before experiments. To determine the effects of epinephrine and AMPK on HSL phosphorylation and activity, cells were subjected to one of the following conditions: 60-min incubation with PBS (V), 60-min incubation with 2 mM of the AMPK-activator AICAR (A), 60-min incubation with 100 nM epinephrine (E), 30-min incubation with AICAR after which epinephrine was added for a further 30 min (AE), or 30-min incubation with epinephrine after which AICAR was added for a further 30 min (EA). Cells were lysed for later analysis of HSL phosphorylation and protein content or HSL activity as described below. Lipolysis in 3T3-L1 was assessed as above (epinephrine concentration 100 nM, duration 60 min) except that cells were incubated in pregassed modified Krebs-Henseleit buffer. An aliquot of the buffer was obtained, and glycerol content was measured as described below. Epinephrine was used at a final concentration of 100 nM to maintain consistency with the phosphorylation experiments, and previous reports demonstrate 1.8- to 3-fold increases above basal lipolysis at this concentration (5, 13).

Analysis

Whole blood was collected into a heparinized tube and placed into separate tubes for later analysis. Blood for glucose, lactate, free fatty acid (FFA), and insulin determination was spun and the plasma stored at −80°C for later analysis. Blood for epinephrine determination was treated with EGTA and reduced glutathione, spun, and stored. Glucose and lactate were immediately analyzed by an automated method (2300 STAT; Yellow Springs Instruments, Yellow Springs, OH). Plasma FFA concentration was determined by colorometric assay (Wako NEFA C test kit; Wako Chemicals, Richmond, VA), insulin by radioimmunoassay (Coat-a-Count insulin test kit; Diagnostics Products, Los Angeles, CA), and epinephrine by radioimmunoassay (Adrenaline RIA, Labor Diagnostika Nord, Nordhorn, Germany).

Muscles were freeze-dried, and nonmuscle contaminants were removed under magnification. Muscle was analyzed for HSL activity as described after immunoinhibition with anti-HSL antibody (29). A second aliquot of muscle was extracted by a Folch extraction, the triacylglycerol was saponified in an ethanol-KOH solution at 60°C, and glycerol content was determined fluorometrically as described (9, 25). A third aliquot of muscle was homogenized (Polytron; Brinkman Instruments, Westbury, NY) in 20 mmol/l HEPES, 1 mmol/l DTT, 1 mmol/l $Na_4P_2O_7$, 2 mmol/l EDTA, 1% Triton X-100, 10% glycerol (vol/vol), 3 mmol/l benzamidine, 1 mmol/l phenylmethylsulfonyl fluoride, 5 ml/l phosphatase inhibitor cocktail 2 (Sigma), and 5 ml/l protease inhibitor cocktail (Sigma) and rotated for 40 min at 4°C. Homogenates were centrifuged at 16,000 *g* for 60 min, and the supernatant was removed and rapidly frozen in liquid nitrogen. Protein concentration of the muscle lysates was subsequently determined according to the bicinchoninic acid method (Pierce Kit; Progen Industries, Darra, QLD, Australia). Muscle lysates (120 mg) were solubilized in Laemmli sample buffer and boiled for 5 min, resolved by SDS-PAGE on 8–12% polyacrylamide gels, transferred to a nitrocellulose membrane, blocked with 5% BSA, and immunoblotted with primary antibody. Phospho-ERK1/2 MAP kinase antibody (1:1,000; Cell Signaling, Beverley, MA) detects endogenous levels of ERK1/2 only when dually phosphorylated at $Thr^{202/204}$. The expression and phosphorylation of acetyl-CoA carboxylase-β (ACCβ) and AMPK were detected by immunoblot from the same muscle lysates with ACCβ-Ser^{221} and AMPK Thr^{172} antibodies, and the antibodies were stripped and reprobed for total ACCβ and AMPKα, respectively, as previously described (3). After incubation with horseradish peroxidase-conjugated secondary antibody (1:2,000; Amersham Biosciences, Castle Hill, NSW, Australia), the immunoreactive proteins were detected with enhanced chemiluminescence (PerkinElmer, Rowville, Australia) and quantified by densitometry (ChemiDoc XRS; Bio-Rad Laboratories, Regents Park, NSW, Australia). Membranes were stripped (50 mmol/l Tris·HCl, pH 6.8, 2% SDS, 2-mercaptoethanol) for 45 min at 55°C, washed, and reprobed for total protein content where appropriate. Adipose tissue samples were treated as described for skeletal muscle except that 100 μg protein was loaded for analysis.

Development of Anti-HSL Antibodies and In Vitro Validation

Rabbit polyclonal antibodies raised against the peptide and phosphopeptides based on the amino acid sequence of human HSL (192–202) EHYKRNETGL (HSL), (557–569) pS-^{563} C^{557} CESMRRpSVSEAAL, (557–569) pS-^{565} C^{557} CESMRRSVpSEAAL, and (653–664) pS-^{660} C^{653} CFHPRRSpSQGVL were purified using procedures described previously (3). The specificity of the HSL antibodies against HSL were checked by in vitro assay. Recombinant rat HSL (generously provided by Prof. Fredric Kraemer, Stanford University and Veterans Administration Palo Alto Health Care System, Palo Alto, CA) was incubated in a buffer consisting of 50 mmol/l HEPES (pH 7.4), 400 μM ATP, 5 mmol/l MgCl, 1 mmol/l DTT, and 0.1% Triton X-100. AMPK (100 ng), PKA (100 U/ml), or no kinase was added, and the incubation proceeded for 30 min at 37°C. Laemmli buffer was added, and the proteins were analyzed by SDS-PAGE and probed with anti-HSL Ser^{563}, Ser^{565}, or Ser^{660} phosphospecific antibody. Blots were stripped and reprobed for total protein content.

Statistical Analysis

Data are expressed as means ± SE. Statistical analysis was performed by one-way analysis of variance (ANOVA) with repeated measures, and specific differences were located using a Student-Newman-Keuls post hoc test. For cell experiments, differences were located using a one-way ANOVA. Statistical significance was set at $P < 0.05$.

Table 1. *Respiratory responses during exercise at 60% $\dot{V}O_{2\ peak}$*

Time, min	$\dot{V}O_2$, ml·kg^{-1}·min^{-1}	RER	V_E, l/min
15	30.2±1.5	0.93±0.01*	55.3±4.8
30	29.9±1.4	0.90±0.01*	52.0±3.8
60	29.4±1.6	0.86±0.01*	51.3±3.3
90	29.5±1.9	0.84±0.01*	53.0±3.8

Values are means ± SE; $n = 8$. $\dot{V}O_2$, oxygen uptake; RER, respiratory exchange ratio; V_E, minute ventilation. *Significantly different from the previous time point.

RESULTS

In Vitro Validation of Phosphospecific HSL Antibodies

Anti-HSL Ser^{563} and anti-HSL Ser^{660} detected HSL when incubated with PKA, whereas anti-HSL Ser^{565} detected HSL when incubated with AMPK (Fig. 1*B*). Reprobing of membranes with anti-HSL revealed uniform loading of HSL protein (Fig. 1*B*). These data are consistent with previous studies demonstrating PKA phosphorylation at Ser^{563} and Ser^{660} and AMPK phosphorylation at Ser^{565} (1, 10, 11, 22).

Human Studies

Respiratory responses and blood chemistry in humans at rest and during exercise. $\dot{V}O_2$ averaged 30.2 ± 1.5 ml·kg^{-1}·min^{-1} at 15 min of exercise and was unchanged throughout exercise (Table 1). The respiratory exchange ratio decreased ($P < 0.05$) with exercise duration (Table 1), whereas minute ventilation did not change throughout exercise (Table 1). Blood glucose was maintained at basal levels throughout exercise and recovery (Table 2), whereas plasma lactate was increased ($P < 0.05$) during exercise and returned to basal concentrations by 120 min after exercise (Table 2). Plasma FFA averaged 0.51 ± 0.05 mmol/l at rest, was increased ($P < 0.05$) by 90 min of exercise, and remained elevated in recovery (Table 2). Plasma epinephrine increased ($P < 0.05$) progressively throughout exercise and returned to preexercise levels during the recovery period (Table 2). Plasma insulin levels were decreased ($P < 0.05$) from resting levels by 15 min and furthermore at 90 min. Insulin levels remained lower 120 min after exercise (Table 2).

Muscle Triacylglycerol Content in Humans Before and After Exercise

IMTG content averaged 20.3 ± 4.8 mmol/kg dry mass at rest and tended to decrease after 90 min exercise (16.8 ± 1.7 mmol/kg dry mass, $P = 0.18$). IMTG content averaged 20.1 ± 1.8 mmol/kg dry mass 120 min after exercise.

Protein Phosphorylation and HSL Activity in Exercising Humans

Skeletal muscle. AMPK Thr^{172} phosphorylation was increased ($P < 0.05$) from rest 1.7- and 2.7-fold at 15 and 90 min, respectively, and returned to resting values 120 min after exercise (Fig. 2*A*). The changes in ACC Ser^{221} phosphorylation essentially replicated AMPK Thr^{172} phosphorylation (Fig. 2*B*). ERK1/2 phosphorylation was increased ($P < 0.05$) from rest at 15 and 90 min of exercise and returned to basal levels 120 min after exercise (Fig. 2*C*). For HSL activity measurements, total neutral lipase activity was determined in muscle lysates exposed to anti-HSL antibody or vehicle. HSL activity was then calculated as total neutral lipase activity minus the activity following anti-HSL antibody pretreatment. Skeletal muscle HSL activity represented ~70% of basal and ~95% of total neutral lipase activity during exercise. HSL activity increased ($P < 0.05$) by 78 ± 9% from rest at 15 min of exercise, returned to preexercise rates by 90 min (Fig. 3*A*), and remained at resting rates 120 min after exercise. Representative immunoblots of HSL serine phosphorylation and total HSL are presented in Fig. 3*B*. The PKA-stimulated sites HSL Ser^{563} and Ser^{660} were increased ($P < 0.05$) by 27% from rest at 15 min (Fig. 2, *C* and *D*). Ser^{563} phosphorylation was increased ($P < 0.05$) further at 90 min, and although Ser^{660} phosphorylation remained elevated at 90 min, this value was not different from 15 min. Both Ser^{563} and Ser^{660} phosphorylation returned to resting levels 120 min after exercise. The phosphorylation of the AMPK-stimulated site Ser^{565} was unchanged from rest at 15 min of exercise and was increased ($P < 0.05$) by 24 and 36% at 90 min of exercise and 120 min after exercise, respectively (Fig. 3*E*). Total HSL protein was not different from rest during or after acute exercise (Fig. 3*B*).

Adipose tissue. AMPK Thr^{172} phosphorylation was increased at 90 min and returned to resting levels 120 min after exercise (Fig. 2*A*). ACC Ser^{221} phosphorylation was variable and tended to be increased from rest at 90 min of exercise (223 ± 79%, $P = 0.15$) and 120 min after exercise (155 ± 27%, $P = 0.19$). ERK1/2 phosphorylation increased modestly during exercise, reaching significance at 90 min (Fig. 2*C*). Phosphorylation of ERK1/2 returned to basal levels after exercise. Adipose tissue HSL was not measured in this study due to tissue limitations; however, we have previously demonstrated that HSL activity remains elevated in adipose tissue throughout 180 min of moderate-intensity cycle exercise (28). HSL Ser^{563} was increased by 51 ± 12% ($P < 0.05$) from rest at 15 min, tended to remain elevated at 90 min, and returned to resting values 120 min after exercise (Fig. 3*C*). HSL Ser^{660} phosphorylation was greater than rest during exercise, reaching significance ($P < 0.05$) at 90 min (Fig. 3*D*). Similar to Ser^{563}, phosphorylation at Ser^{660} returned to resting levels after

Table 2. *Blood metabolite and hormone responses before, during, and 2 h after 60 min of cycle exercise at 60% $\dot{V}O_{2\ max}$*

Time, min	Glucose, mmol/l	Lactate, mmol/l	FFA, mmol/l	Epinephrine, nmol/l	Insulin, pmol/l
0	4.76±0.15	0.97±0.11	0.51±0.05	0.30±0.07	37±2
15	4.73±0.24	3.60±0.68*	0.43±0.03	0.72±0.11*	27±3*
90	4.46±0.09	2.04±0.31*	0.70±0.07*	2.15±0.46*†	14±4*
120 min post	4.56±0.09	1.15±0.12	1.15±0.24*	0.30±0.04	18±3*

Values are means ± SE; $n = 8$. FFA, free fatty acid. *Significantly different from 0 min; †significantly different from all other times.

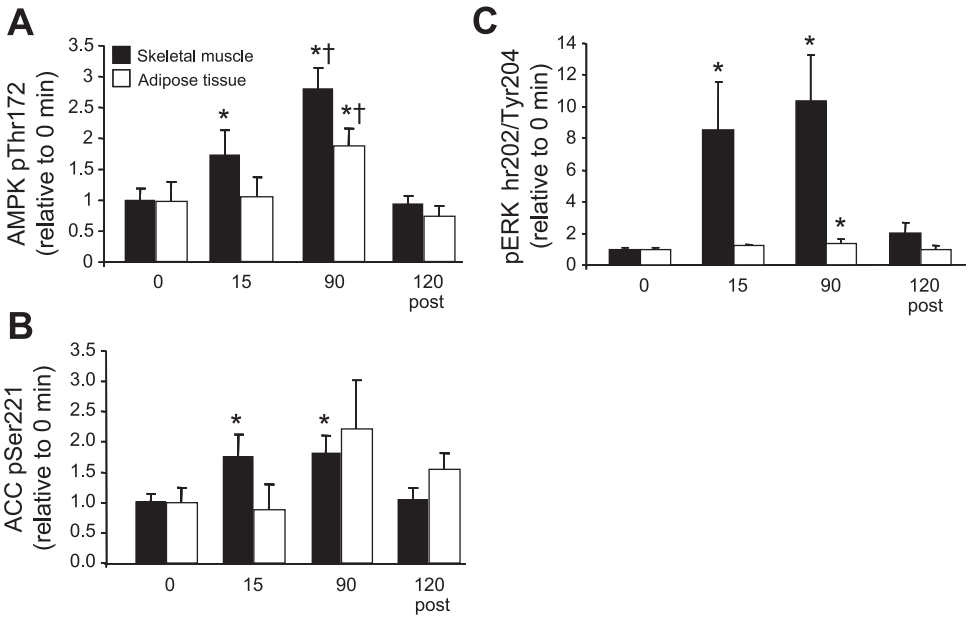


Fig. 2. Skeletal muscle and adipose tissue AMPK Thr^{172} (*A*), acetyl-CoA carboxylase (ACC) Ser^{221} (*B*), and ERK1/2 (*C*) phosphorylation before, during, and after 90 min of cycle exercise at 60% of peak O_2 uptake. Values are normalized to total AMPKα and ACCβ and total protein content for ERK1/2. Protein content is expressed relative to the value of 0 min. *Different from rest; †different from all other time points ($P < 0.05$).

exercise. HSL Ser^{565} was elevated at 90 min of exercise and after exercise ($P < 0.05$; Fig. 3*E*).

Regulation of HSL Phosphorylation and Activity in Cell Culture

Skeletal muscle myotubes. AMPK Thr^{172} and HSL Ser^{565} phosphorylation were increased by AICAR treatment irrespective of the presence of epinephrine in the incubation media (Fig. 4, *A* and *B*). HSL activity in L6 myotubes was increased ($P < 0.05$) by epinephrine, and AMPK activation by AICAR blunted this effect (Fig. 4*C*). Phosphorylation of the PKA sites HSL Ser^{563} and Ser^{660} was increased by epinephrine (Fig. 4, *E* and *F*), whereas prior activation of AMPK was associated with reduced HSL Ser^{660} but not Ser^{563} phosphorylation. These data indicate that HSL Ser^{660} phosphorylation correlates with HSL activation in skeletal muscle cells and that prior activation of AMPK can inhibit HSL Ser^{660} phosphorylation and HSL activity. In separate experiments, infecting a CA-AMPK into L6 myotubes inhibited HSL activity by 40% (Control 0.70 ± 0.05 vs. CA-AMPK 0.42 ± 0.08 nmol·min^{-1}·mg protein^{-1}), thereby confirming AMPK inhibitory effects on skeletal muscle HSL activity.

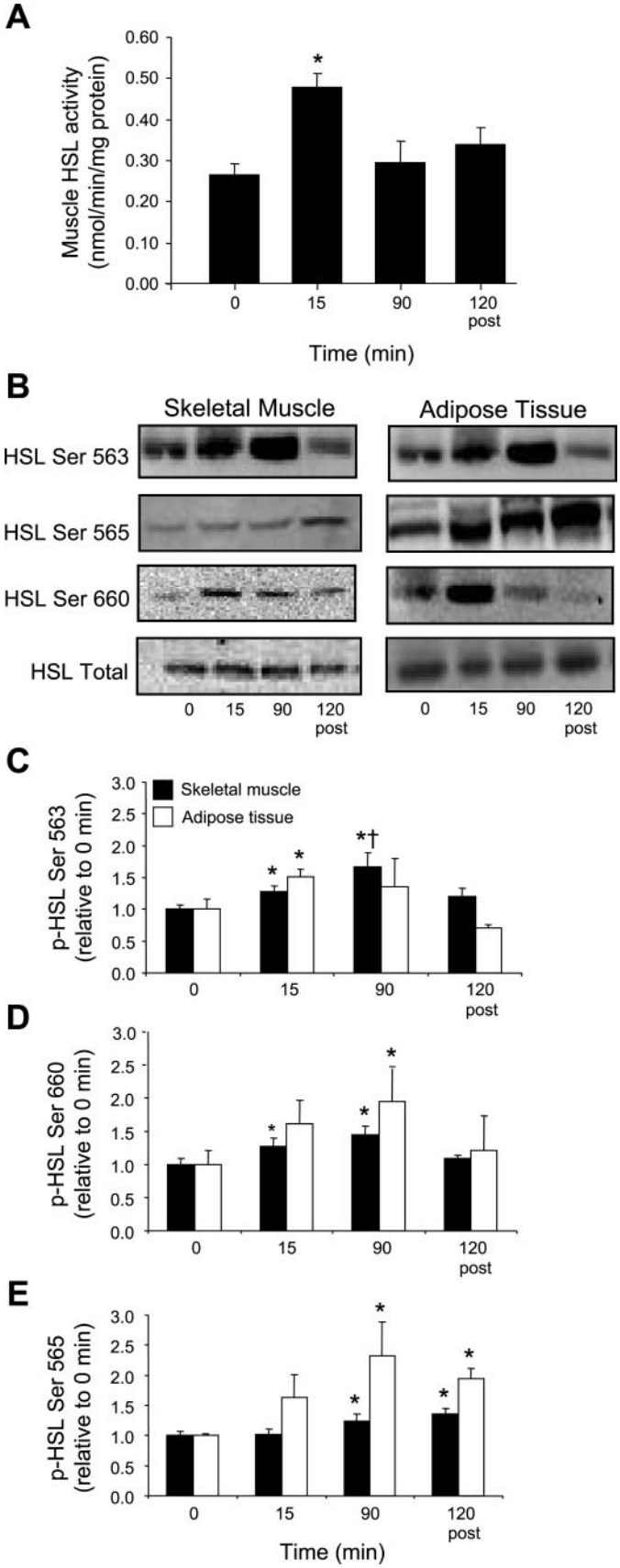
Adipocytes. AMPK Thr^{172} phosphorylation was not increased by epinephrine (Fig. 4*A*), which contrasts with previous reports (17, 34). HSL phosporylation at Ser^{565} paralleled AMPK activation, with the exception of the AE condition ($P = 0.12$; Fig. 4*B*). HSL activity (Fig. 4*C*) and lipolysis (Fig. 4*D*) were elevated above vehicle with epinephrine treatment and when epinephrine administration preceded AMPK activation. However, when AMPK activation preceded epinephrine administration, both HSL activity and lipolysis were attenuated. HSL Ser^{563} and Ser^{660} were increased with epinephrine (Fig. 4, *E* and *F*). Concomitant incubation with AICAR and epinephrine completely suppressed HSL Ser^{563} phosphorylation irrespective of the order in which treatments were added (Fig. 4*E*). HSL Ser^{660} phosphorylation was blunted when AMPK activation preceded epinephrine treatment, whereas high levels of phosphorylation were maintained on HSL Ser^{660} when epinephrine preceded AMPK activation (i.e., EA; Fig. 4*F*). These data indicate that, in contrast to skeletal muscle myotubes, when HSL Ser^{660} is initially phosphorylated in adipocytes HSL activity and lipolysis are increased despite AMPK activation and HSL Ser^{565} phosphorylation.

DISCUSSION

The signaling pathways associated with hormonal and skeletal muscle contractile regulation of HSL activity are poorly understood. Here, we show that the PKA-activated sites HSL Ser^{563} and Ser^{660} are phosphorylated during exercise when plasma epinephrine concentrations are elevated. HSL Ser^{565}, which is proposed to prevent the increase in HSL activity (11), was phosphorylated at 90 min of and in recovery from exercise. The increased AMPK phosphorylation and Ser^{565} phosphorylation at 90 min corresponded to decreased HSL activity, consistent with previous results suggesting that phosphorylation of HSL by AMPK prevents activation of this enzyme (11, 29). In contrast, HSL activity is maintained in adipose tissue during prolonged exercise, suggesting that phosphorylation at HSL Ser^{660} can overcome AMPK effects in this tissue, an interpretation supported by studies in 3T3-L1 adipocytes.

Control of Skeletal Muscle HSL Activity

Skeletal muscle HSL activity increased early in exercise, as observed previously in exercising humans (20, 27). The increased HSL activity occurred concomitantly with elevated plasma epinephrine and greater phosphorylation of the PKA regulatory sites HSL Ser^{563} and Ser^{660}, which is consistent with a β-adrenergic-mediated increase in HSL activity early in exercise. Of note, in a previous study, HSL activity during exercise was reduced in adrenalectomized humans and was restored when epinephrine was replaced (14), supporting our data that phosphorylation of HSL at Ser^{563} and Ser^{660} is mediated by β-adrenergic stimulation. The importance of the PKA catalytic sites has not been fully resolved because studies



that have implemented site directed mutagenesis against HSL Ser^{563} have reported either no effect (1) or an ~80% reduction (22) of cAMP-dependent enzyme activity against a triacylglycerol substrate. Ser^{659} and Ser^{660} appear to be the major HSL activity-controlling sites (1). Previously, it was reported that exercise did not affect skeletal muscle HSL Ser^{563} phosphorylation in subjects with very low or high preexercise muscle glycogen content despite elevated plasma epinephrine (20). However, we find HSL Ser^{563} phosphorylation with exercise. The reason for these discrepant findings are not clear, but our finding of increased Ser^{563} phosphorylation and a clear in vitro effect supports the idea that Ser^{563} is a target for PKA.

ERK phosphorylates HSL Ser^{600} and increases HSL activity, as demonstrated in 3T3-L1 adipocytes (12) and isolated rodent skeletal muscle (7). ERK phosphorylation was increased 8.5-fold at 15 min of exercise and may have contributed to the increased HSL activity. A corresponding anti-phospho-Ser^{600}-specific antibody was not available to evaluate Ser^{600} phosphorylation in this study.

Phosphorylation at HSL Ser^{565}, which is a substrate for AMPK, prevents HSL activation (11, 29). HSL activity declined to resting rates late in prolonged exercise (90 min) despite high circulating epinephrine concentrations and further increases in the phosphorylation on the PKA site Ser^{563}, which would be expected to increase HSL activity (14, 16, 30). These data are consistent with previous observations in skeletal muscle. Increasing AMPK activity during exercise by lowering muscle glycogen content prevents the exercise-induced increase in HSL activity (29). Adding AICAR to contracting rat soleus muscles inhibits triacylglycerol hydrolysis (23), and incubating L6 myotubes with AICAR inhibits β-adrenergic-stimulated HSL activity (6, 24, 29). The in vitro data from the present study extends these observations by demonstrating that phosphorylation of HSL Ser^{565} by AMPK attenuates HSL activity by blocking or preventing increases in HSL Ser^{660} phosphorylation. The finding of reduced HSL Ser^{660} phosphorylation when L6 muscle cells were incubated with epinephrine followed by AICAR was unexpected, and we are unable to provide an explanation for the reduction at this point. This finding is consistent with the overall interpretation that AMPK overcomes epinenphrine effects in muscle and prevents HSL Ser^{660} phosphorylation and HSL activation. These data in skeletal muscle culture also suggest that HSL Ser^{565} phosphorylation does not prevent HSL Ser^{563} phosphorylation; however, phosphorylation of HSL Ser^{563} does not appear to activate HSL activity.

Although we were unable to measure AMPK activity due to the small size of tissue biopsies, AMPK Thr^{172} and ACCβ Ser^{221} phosphorylation, which closely reflect AMPK activity

Fig. 3. Skeletal muscle and adipose tissue HSL phosphorylation before, during, and 120 min after 90 min of moderate-intensity cycle exercise. *A*: HSL activity represents total neutral lipase activity minus neutral lipase activity after lysates were preincubated with anti-HSL antibody. *B*: representative immunoblots for total HSL and HSL phosphorylated at Ser^{563}, Ser^{565}, and Ser^{660} before, during, and 2 h after 90 min of moderate-intensity cycle exercise. Proteins were isolated from skeletal muscle (120 μg) and adipose tissue (100 μg) lysates. In separate analysis, lysates were probed with antibodies raised against HSL Ser^{563} (*C*), Ser^{660} (*D*), and Ser^{565} (E). Data for phosphoproteins are normalized to total HSL protein and expressed relative to preexercise levels. *Different from 0 min; †different from all other measurements ($P < 0.05$).

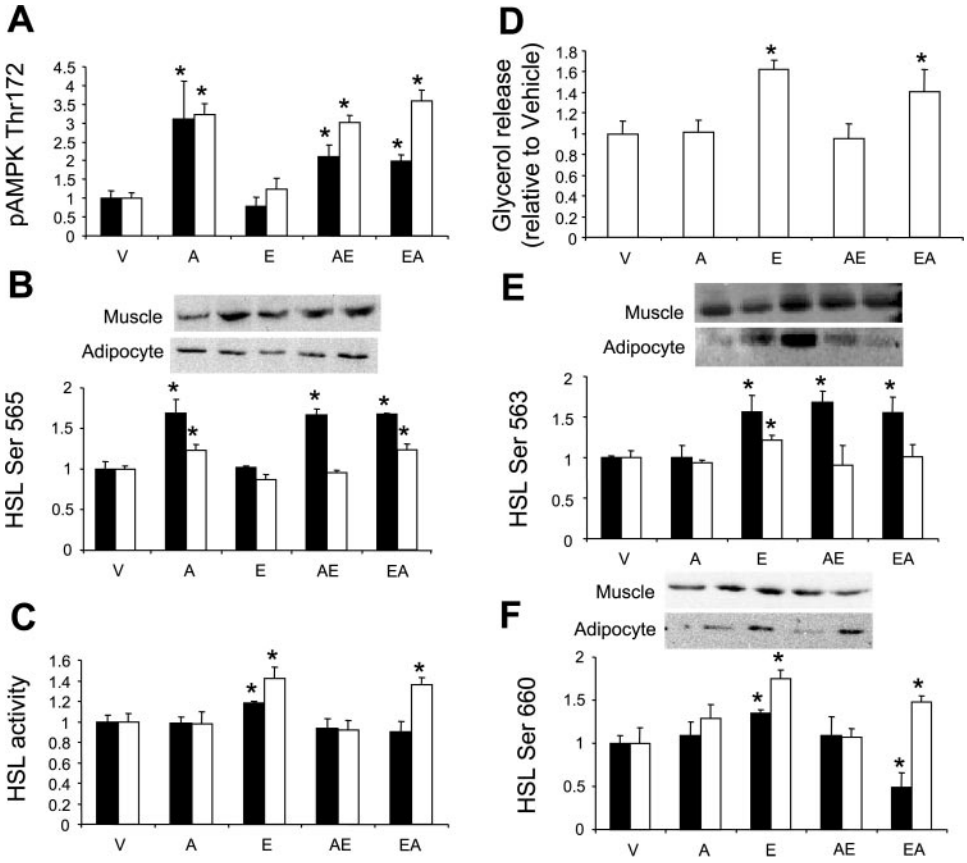


Fig. 4. HSL activity and HSL serine phosphorylation in L6 myotubes (filled bars) and 3T3-L1 adipocytes (open bars) in response to β-adrenergic and AMPK stimulation. V, incubation with PBS vehicle; A, 60-min incubation with 2 mM 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR); E, 60-min incubation with 100 nM epinephrine; EA, 30-min incubation with epinephrine after which AICAR was added for a further 30 min; AE, 30-min incubation with AICAR after which epinephrine was added for a further 30 min. *A*: AMPK Thr^{172} phosphorylation. *B*: HSL Ser^{565} phosphorylation. *C*: HSL activity, which represents total neutral lipase activity minus neutral lipase activity after lysates were preincubated with anti-HSL antibody. *D*: lipolysis represented by glycerol release from 3T3-L1 adipocytes into the medium. *E*: HSL Ser^{563} phosphorylation. *F*: HSL Ser^{660} phosphorylation. For protein analysis, membranes were stripped, and equal loading of total HSL or AMPK protein was confirmed. Representative immunoblots are shown above figures in the same order as listed on the respective *x*-axis. *Different from all other time points ($P < 0.05$).

(4, 19), were temporally related to HSL Ser^{565} phosphorylation late in exercise only (Pearsons correlation at 90 min, $r^2 = 0.65$; for all sampling times, $r^2 = 0.0025$). In contrast to the findings above, Roepstorff et al. (20) reported that increased Ser^{565} phosphorylation had no effect on HSL activity in exercising humans. In the present study, Ser^{565} was not increased at 15 min despite phosphorylation of AMPK Thr^{172} and the downstream target ACCβ Ser^{221}. This indicates a lag in HSL Ser^{565} phosphorylation possibly due to different affinities for the AMPK substrates ACCβ and HSL. Higher levels of AMPK activation at 90 min of exercise were associated with phosphorylation of Ser^{565}, indicating that a threshold of AMPK activation or possible translocation is required for HSL Ser^{565} phosphorylation. Our finding of increased HSL Ser^{565} phosphorylation after exercise despite a return of AMPK activation to preexercise levels indicates that dephosphorylation of this site is delayed during recovery from exercise. Collectively, these data demonstrate that PKA phosphorylation of HSL Ser^{563} and Ser^{660} is associated with greater HSL activity. However, activation of HSL Ser^{565} by AMPK reduces HSL activity in skeletal muscle despite maintenance of PKA-mediated phosphorylation.

Control of Adipose Tissue HSL Activity

Plasma fatty acids derived from adipose tissue triacylglycerol lipolysis are an important energy substrate for resting and contracting muscle (21). The regulation of adipose tissue lipoylsis is incompletely understood, in particular the hierarchy of control at serine residues by various protein kinases. Although PKA stimulates HSL Ser^{563} and Ser^{660} phosphorylation and activates HSL and lipolysis, the role of AMPK in these processes remains controversial. Studies that pretreated rat adipocytes with AICAR demonstrated a reduced lipolytic response to β-adrenergic agonists (6, 24), whereas another study using adenoviral transfection of dominant negative AMPK into 3T3-L1 adipocytes reported that cAMP can lead to activation of AMPK, which is essential for maximal activation of lipolysis (34). The present studies conducted in 3T3-L1 adipocytes demonstrate that β-adrenergic stimulation phosphorylates HSL Ser^{563} and Ser^{660}, and this coincides with HSL activation and lipolysis. When AMPK activation and phosphorylation of HSL Ser^{565} preceded the addition of epinephrine, these effects were completely antagonized, i.e., HSL Ser^{563} and Ser^{660} phosphorylation was blocked and HSL activity attenuated. However, in contrast to skeletal muscle, when epinephrine was added before AMPK activation, HSL Ser^{660} but not HSL Ser^{563} phosphorylation was maintained, and HSL activity was elevated 50% above control. These data indicate that *1*) HSL Ser^{565} phosphorylation prevents HSL Ser^{563} phosphorylation as previously suggested (10), *2*) HSL Ser^{565} phosphorylation can prevent HSL Ser^{660} phosphorylation when AMPK activation precedes PKA stimulation, and *3*) HSL Ser^{660} is an important site for HSL catalytic activity that can override AMPK signaling in adipocytes. Thus both PKA and AMPK are important regulators of HSL serine phoshorylation, HSL activity, and lipolysis, and the current conclusions are important when the control of lipolysis in various physiological settings is considered.

Reversible phosphorylation is a hallmark of HSL control, and the role of exercise on adipose HSL serine phosphorylation

in vivo is unknown. We have demonstrated increased Ser^{563} and Ser^{660} phosphorylation during exercise, which is consistent with the increased plasma epinephrine, a twofold increase in HSL activity (28), and elevated adipose tissue lipolysis (31). Ser^{565} phosphorylation on adipose HSL was also elevated at 90 min. In contrast to the control of skeletal muscle HSL, where regulation at Ser^{565} seems to override control of the PKA sites, HSL activity remains elevated (28) despite increased Ser^{565} phosphorylation. These findings are entirely consistent with our in vitro studies and may reflect the hierarchy of fat utilization late in prolonged exercise where oxidation of plasma-derived FFA seems to predominate over IMTG (25).

In conclusion, the present study demonstrates a number of important roles for tissue-specific control of HSL activity by serine phosphorylation. Our study indicates that *1*) simultaneous phosphorylation occurs at numerous serine sites in skeletal muscle and adipose tissue both in vitro and during exercise in vivo; *2*) HSL Ser^{563} is unlikely to be important for HSL catalytic activity in skeletal muscle; and *3*) AMPK phosphorylation of HSL Ser^{565} anatagonizes β-adrenergic activiation of HSL in skeletal muscle, whereas when β-adrenergic stimulation precedes AMPK activation in adipocytes, HSL Ser^{660} phosphorylation, HSL activity, and lipolysis are maintained. These data highlight the tissue-specific differences in HSL regulation and explain, in part, the maintenance of adipose tissue, but not skeletal muscle, lipolysis during prolonged exercise.

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